Validation Plan for the EDSP Mammalian Tier 2 Assay

Presentation to the
Endocrine Disruptor Methods Validation Subcommittee
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Overview of presentation

- Validation plan
- Concerns
- Questions for EDMVS

Validation plan

- Accept the OPPTS Guideline for Reproductive Toxicity, with additional thyroid endpoints, as valid for EDSP Tier 2 purposes.
- Include clarifications of certain procedures and endpoints already in the Guideline as part of the EDSP assay.
- Ask for independent peer review of the validity of the Guideline and clarifications for EDSP purposes.

Additional plans

- Encourage one-generation extension of F1 when indicated by Tier 1, and in all Tier 1 bypasses.

- Develop additional information on the relevance and reliability of the one-generation extension, for possible future inclusion in the EDSP assay.

The 1998 Reprotox Guideline is generally accepted as valid…

- … for regulatory risk assessment of reproductive toxicity
- Reproductive toxicity can be “an effect produced by a naturally occurring estrogen” and can be an indicator of endocrine effects
- ∴ the Guideline is valid for EDSP purposes.

Additional endpoints

- EDSTAC: consider specific additional endpoints, for non-reprotox endpoints
- SVTF: adding all of these would be impractical and in some cases not useful; focus on thyroid-specific endpoints
- Relevance of most of the thyroid-specific endpoints was shown in the PTU study which EDMVS reviewed (but drop T_3)

Table 1: Additional endpoints proposed for EDSP purposes

TSH, T4, thyroid weight, thyroid histology, all at necropsy	Not in current Guideline	Declined to add T3 on the basis of PTU study results. Add – or change – T4/TSH measurement to PND 21?
Areola/nipples: what, where, how many, in both males and females, F1 and F2, PND 13. Also at necropsy for F1 only.	"At the time of termination or death during the study, all parental animals (P and F1) and when litter size permits at least three pups per sex per litter from the unselected F1 weanlings and the F2 weanlings should be examined macroscopically for any structural abnormalities or pathological changes. Special attention should be paid to the organs of the reproductive system."	Differences are: addition of a new timepoint for examination (pnd 13); and number of animals (all, vs. 3/sex/litter).
Anogenital distance, all animals in both F1 and F2, at birth (PND 2)	AGD only for F2 and only when triggered by a treatment-related effect in F1 sex ratio or sexual maturation. At birth (PND 0)	
Whole-mount histology of mammary tissue in males, triggered if abnormalities are seen in gross examination.	"For F1 and F2 weanlings, histopathological examination of treatment-related abnormalities noted at macroscopic examination should be considered, if such evaluation were deemed appropriate and would contribute to the interpretation of the study data." (Whole-mount not specified)	

Clarifications to current Guideline

- Table 2: Specification of endpoints for mammalian two-generation study" (in handout)

Table 2: Specification of endpoints for mammalian two-generation study

The following specific endpoints are already covered by the current Reproductive Toxicity Guideline in the sections quoted in Table 1 above, but are listed for greater clarity and to ensure adequate attention to important details.

EDSTAC suggested that it may be appropriate to "tailor" Tier 2 tests if previously collected information of appropriate quality indicates no interaction with one or more of the endocrine systems examined in the Endocrine Disruptor Screening Program[5]. Using EDSTAC's example: in cases where Tier 1 results are available and weight-of-the-evidence evaluation indicates that interaction with the thyroid system is unlikely, it might be acceptable, for EDSP purposes, to forego the collection of information related solely to thyroid effects in the Tier 2 mammalian assay. The following list of clarifications may be an appropriate starting point when such tailoring is being considered.

Suggestions for portions of in-life and necropsy procedures are included to show how important endpoints can be measured.

MALES:

Necropsy after puberty

1. body weight, any unusual malformations or anomalies, euthanize
2. shave ventral surface from inguinal region to neck and count nipples and areolas (observer blind to treatment), record position of areolas and nipples
3. check animals for hypospadias, epispadias, cleft phallus and measure AGD
4. note if testes obviously undescended
5. note if inguinal region soiled with urine
6. note if prepuce partially or entirely detached from glans penis, especially if a persistent thread of tissue is present along frenulum
7. for animals on study past puberty, record age at onset of preputial separation and age at complete PPS (if different). Also record weights at PPS.
8. Weights of animals after weaning, at least twice a week.

Internal endpoints

9. location of each testis (scrotal, abdominal, gubernaculum attached to abdominal wall)
10. gubernacular cords, present or absent, and length in mm
11. note if present, cranial suspensory ligaments
12. note if testes are small, absent, fluid filled, enlarged, appear infected or other
13. note if epididymides are small, absent, or infected (record region of effects)
14. note if ventral prostate is small, absent or infected

[5]Op. cit., p. 5-47

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15. note if dorsolateral prostate is small, absent, or infected
16. note if seminal vesicles are small, absent, infected, or one side larger than the other
17. note if coagulating glands are small absent, infected, one side larger than the other, or detached from seminal vesicles
18. note if kidneys display hydronephrosis, calcium deposits
19. note presence of hydroureter
20. note presence of bladder stones or blood in bladder

Weights and histology

21. each testis individually (histo for both or one for sperm numbers and one histo)
22. each corpus plus caput epididymis (histo for both or one for sperm numbers and one for histo)
23. each cauda epididymis (histo for both or one for sperm numbers and one for histo)
24. entire seminal vesicle, plus coagulating glands with fluid as a unit, if possible
25. entire ventral prostate, if possible (histo)
26. each kidney
27. paired adrenals
28. liver
29. levator ani plus bulbocavernosus
30. Cowper's glands as a pair, if possible
31. glans penis
32. dorsolateral prostate (histo)
33. brain
34. pituitary
35. thyroid weight after fixation and histology
36. heart weight and histo if the chemical is suspect antithyroid

FEMALES

Necropsy

37. body weight, any unusual malformations or anomalies, euthanize
38. shave ventral surface from inguinal region to neck and count nipples and areolas (observer blind to treatment), record position of areolas and nipples
39. check animals for female rat hypospadias, cleft phallus and measure AGD, AVD
40. note if vaginal opening not present.
41. note if vaginal thread is present
42. note if mammary tumors are present (histo if present)

Note: females can have the estrous cycle staged and all killed on the same day of estrous, but if not a terminal vaginal smear should be taken to distinguish proestrus (when the uterus is large) from other stages (when it is smaller)

Internal observations

43. position, size and color of ovaries
44. presence of cranial suspensory ligaments
45. presence of follicular cysts on ovary or atrophy of ovary

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46. absence of lower vagina
47. uterine abnormalities, including bi- or unilateral agenesis of oviducts of uterine horns, infections, hydrometrocolpos, etc.
48. presence of any male tract tissues, including ventral prostate, seminal vesicles, Cowper's glands, levator ani or bulbocavernosus muscles. (Save any for histological confirmation)

Necropsy weights and histology

49. body, liver, kidneys, adrenals, brain, pituitary, heart (if antithyroidal) weights and histology on abnormalities.
50. ovaries (histo)
51. oviducts (histo not weight, if abnormal)
52. uterine weight and histology

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Concerns

- Strain/species White Paper (August)
- Interlab variability
- Thyroid: other mechanisms; sensitivity
- Extension of F1: additional studies
- Timing, if other studies are needed

Questions for EDMVS #1 of 5

- Does the EDMVS agree that the additional endpoints/clarifications proposed for the 2-generation assay (Table 1) are well-characterized and that further validation of this set of endpoints for use in EDSP Tier 2 is unnecessary?

Questions for EDMVS #2 of 5

- Does the EDMVS agree that the endpoints in the Tier 2 assay will allow a compound to be identified as possibly having “an effect in humans that is similar to an effect produced by a naturally occurring estrogen” (or androgen/anti-androgen or thyroid mimic/inhibitor) *in the absence of Tier 1 data*? If not, what other endpoints should be included, or what supplemental testing would be appropriate?

Questions for EDMVS #3 of 5

- Does the EDMVS agree that the procedures and endpoints in Table 2 of the handout should be listed explicitly, to ensure adequate examination?

Questions for EDMVS #4 of 5

- If EDMVS advises EPA to validate additional endpoints,
  - can “new” endpoints be validated separately from endpoints already in the reproductive toxicity assay?
  - is it necessary to validate all new endpoints in a *two*-generation study, or can relevance and reliability be established in a shorter assay
  - how many laboratories should be required for interlaboratory comparability?
  - how many chemicals per mode of endocrine activity should be tested in validation? (e.g., ER/AR binding, each step of steroidogenesis, thyroid hormone transport protein binding, thyroid hormone metabolism, etc.)

Questions for EDMVS #5 of 5

- Does the EDMVS agree that the one-generation extension study shows increased sensitivity and provides greater precision in dose/response assessment, which will be of use in risk assessment, when the F1 animals are allowed to mature to PND 95 than when they are sacrificed at PND 21?